The Mucoprotein of the Fat/Plasma Interface of Cow's Milk

A mucoprotein isolated from the fat/plasma interface of cow's milk has been characterized. As analyzed by free-boundary electrophoresis, the mucoprotein was essentially homogeneous with respect to surface charge density. Chemically, it contained considerably higher levels of sialic acid, hexose, and hexosamine than any of the major proteins of milk. The strong tendency of this substance to aggregate in high gravitational fields was absent in sedimentation experiments using reduced centrifugal speeds.

INTRODUCTION

In 1955 King (1) reviewed the extensive literature on the existence and chemical nature of the stabilizing layer or membrane that coats the fat globule in cow's milk. The existence of this membrane was postulated by Ascherson in 1840 and was demonstrated microscopically by Storch in 1897 with the aid of staining techniques. Later workers extended these observations by the use of dark-field illumination and more recently by means of electron microscopy [cf. (1)].

The separation of interfacial material has been accomplished by many workers. Palmer and associates [cf. (1)], in classical studies of the fat globule membrane, elaborated the following general method for separating the interfacial complex from butter fat and other components of milk. Fresh milk is separated, and the cream is washed repeatedly with water to free the fat globules of dispersed constituents of milk. The washed cream is then churned, and the interfacial material is recovered from the buttermilk and butter plasma. Protein, phospholipids, and a high-melting triglyceride fraction, as well as other minor constituents, have been identified as components of the complex [cf. (1)]. The interfacial protein has never been well defined, but has usually been found to exhibit properties unlike those of other known milk proteins.

The method of recovery of the water-soluble protein used in the present study is based on the technique outlined by Herald and Brunner (2). It consists essentially of precipitation of buttermilk from washed cream by ammonium sulfate, removal of fatty and phospholipid components with alcohol and ether, dispersion in water, dialysis, and lyophilization. The material isolated is a mucoprotein that is distinctly different in composition from the known proteins of milk. Jackson and Pallansch (3) have demonstrated that it exhibits high interfacial activity, markedly greater than that of the caseins and whey proteins.

The isolation of a mucoprotein from the interface confirms, in part, the theory of Storch (4) who proposed that the fat globules were surrounded by a special mucoid substance.

[1] Presented at the 140th Meeting of the American Chemical Society, Chicago, Ill., September, 1961.

[2] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

EXPERIMENTAL

Isolation of the Mucoprotein

One hundred gallons of pooled herd milk was separated at 40°C. with a DeLaval separator, model 619,[3] within 3 hr. after milking. The cream, containing 30–35% butterfat, was washed four times by stirring gently with 3 vol. water at 40°C. for 5 min. and reseparating the cream. The washed cream was chilled over a surface cooler to 13–15°C. and churned immediately in a 15-gal. aluminum churn. The buttermilk was drawn off and passed twice through the separator at 13–15°C. to remove excess fat. Solid $(NH_4)_2SO_4$ was stirred into the buttermilk to a concentration of 2.2 *M*, and the suspension was allowed to stand overnight. The floc was recovered by centrifugation at 25,000 × *g* for 30 min. The precipitate, containing 34–36% solids, was gently agitated for 15 min. at 0° to −5°C. in a solvent mixture consisting of 35% ethyl alcohol in ether, using 4 ml. of the solvent per gram of precipitate. The residue was collected by filtration on Whatman No. 1 filter paper and washed five times with 200 ml. of cold ether. The residue was then extracted three times, for a period of 10 min. each, with ether at room temperature. The defatted residue was made into a slurry with a minimal volume of distilled water, and the residual solvent was evaporated under vacuum. Five volumes of distilled water was added to the slurry and gently stirred for 30 min. The suspended solids were removed by centrifugation at 25,000 × *g* for 30 min. The supernatant, containing the mucoprotein, was dialyzed for 3 days against distilled water and then lyophilized. The yield was 6–8 g. protein.

The quantity of milk used here necessitates the use of pilot-plant scale equipment in the initial steps. Small preparations have been made successfully by using laboratory-scale apparatus.

Since the method of isolation used in this study was based on techniques outlined by Herald and Brunner (2), it is assumed that the interface mucoprotein is one of the components of their "soluble membrane fraction."

Physical Measurements

Free-boundary electrophoretic data were obtained using an Aminco portable electrophoresis unit. Ultracentrifugal experiments were performed with a Spinco model E analytical centrifuge.

Paper Chromatography

Descending, unidimensional paper chromatograms on Whatman No. 1 filter paper were run for 18 hr. in a butanol–acetic acid–water solvent system (50:12:25). Aniline hydrogen phthalate (5) and the Elson-Morgan sprays (6) were used as color-developing reagents for hexose and hexosamine, respectively. Qualitative identification was made using standard sugars spotted as controls.

[3] Mention of products in this paper does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

Enzymic Digestion

The mucoprotein was digested with papain (30:1) in a solution which was 0.005 *M* with respect to cysteine and 0.005 *M* with respect to ethylenediamine tetraacetate (EDTA). The digestion continued for 96 hr. at 37°C. after which the digest was dialyzed and passed over a column of Sephadex-25 gel (1 × 15 cm.). The carbohydrate-containing fraction was then passed over a column of Sephadex-50 gel (1 × 15 cm.) and lyophilized. Aliquots of fractions were analyzed for carbohydrate on a spot plate using a modified Molisch test.

Chemical Analyses

Hexose. Protein-bound hexose was measured by a slightly modified method described by Lustig and Langer (7) as employed by Weimer and Moskin (8). The method is presented in the review by Winzler (9). A mannose–galactose standard was used in each analysis.

Hexosamine. The method of Elson and Morgan (10) was used. Appropriate concentrations of glucosamine hydrochloride standards were included in each determination.

Methyl Pentose. The method of Dische and

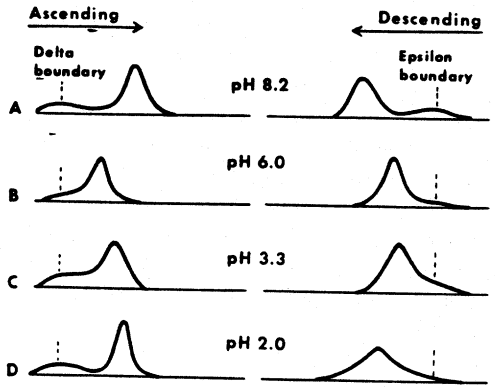

Fig. 1. Free-boundary, electrophoretic patterns of the mucoprotein at various pH values. Protein concentration, 2.0%; buffers of 0.1 ionic strength; time, 8000 sec.; and current, 15.0 ma. Broken lines indicate initial boundaries. *A*, Veronal buffer, $\mu = -4.6$; *B*, phosphate buffer, $\mu = -2.8$; *C*, glycine-HCl buffer, $\mu = 2.0$; *D*, glycine-HCl buffer, $\mu = 5.1$. $\mu = 1 \times 10^{-5}$ sq. cm./v./sec.

Shettles (11) was used. Fucose or rhamnose standards (10 μg.) were included in each analysis.

Uronic Acid. This determination was made according to the technique of Dische (12). Standards of glucuronic acid were used in each determination.

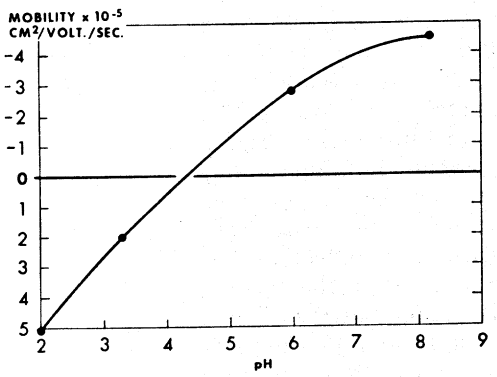

FIG. 2. pH–mobility curve of the mucoprotein.

Sialic Acid. This constituent was determined by three methods: the thiobarbituric acid method of Warren (13), the diphenylamine technique of Ayala *et al.* (14) as presented in slightly modified form by Winzler (9), and with Bial's orcinol reagent in a method suggested by Papadopoulos and Hess (15). Appropriate standards of crystalline *N*-acetylneuraminic acid isolated from bovine colostrum were included in each determination.

Reducing Sugar. Reducing sugar was measured by the method of Folin and Wu (16) after hydrolyzing with 1 *N* H_2SO_4 for 6 hr. at 100°C.

RESULTS AND DISCUSSION

Free-boundary electrophoretic experiments with the mucoprotein were conducted

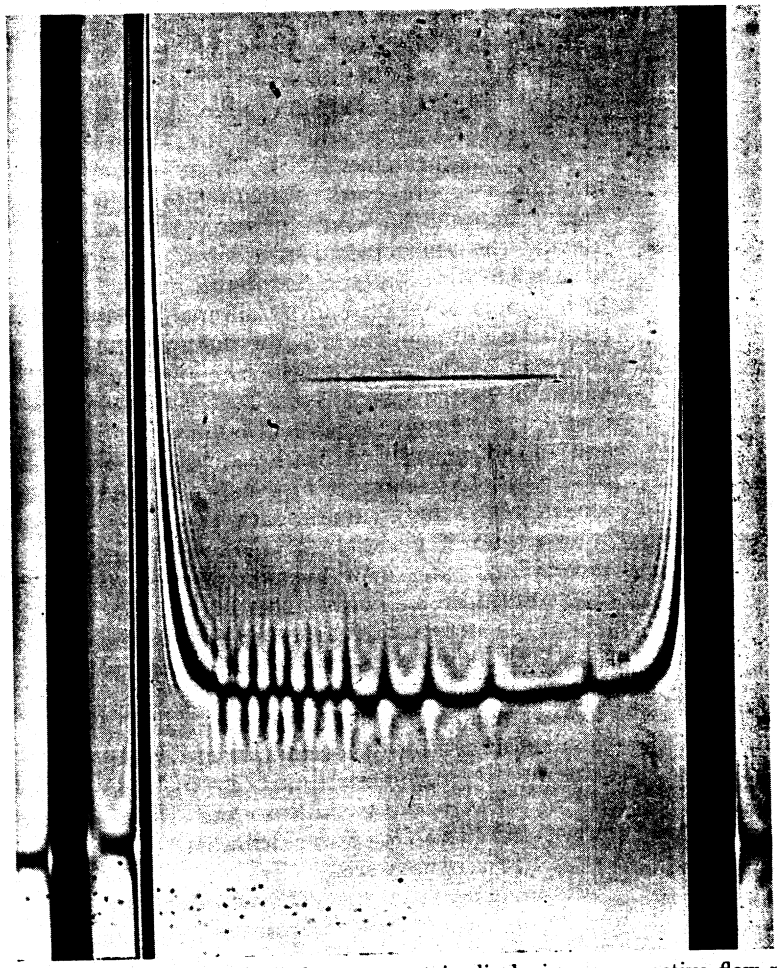

FIG. 3. Sedimentation pattern of the mucoprotein displaying a convective flow phenomenon at high centrifugal speed (59,780 r.p.m.).

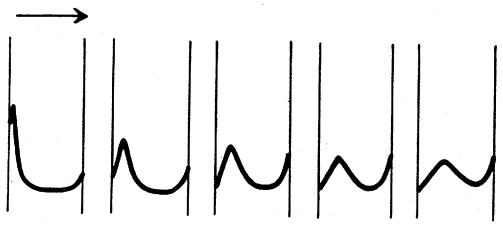

FIG. 4. Sedimentation pattern of the mucoprotein at a low centrifugal speed.

TABLE I

CHEMICAL COMPOSITION OF THE INTERFACE MUCOPROTEIN

	Percentage
Hexose	5.2–5.9
Hexosamine	3.9
Sialic acid:	
Diphenylamine reagent	4.6–4.8
Bial's reagent	4.0
Uronic acid	<1.0
Methyl pentose	<1.0
Reducing sugar	8.3
Nitrogen	11.28
Phosphorus	0.68
Ash	6.12
Lipid	2.1

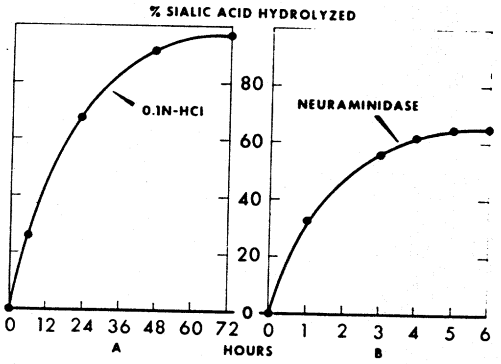

FIG. 5. Per cent sialic acid hydrolyzed from the mucoprotein by neuraminidase and dilute acid.

over the pH range 2.0–8.2 as shown in Fig. 1. There was only one boundary in both the descending and ascending channels, and the patterns simulated homogeneous Gaussian curves. The only noticeable deviation was at pH 2.0 in the descending channel where the boundary had a tendency to diffuse and the pattern became slightly asymmetrical. These electrophoretic analyses were continued for periods of approximately 4 hr. At the end of this time, the boundaries were diffuse but never indicated a tendency to separate. The isoelectric point was 4.2 as taken from the pH–mobility curve, Fig. 2.

Difficulties were encountered in obtaining ultracentrifugal data for the mucoprotein. As the full speed (59,780 r.p.m.) of the centrifuge was approached, convective-flow disturbances began to disrupt the leading edge of the sedimentation pattern. These disturbances were manifested in the patterns as saw-tooth spikes which indicate extremely sharp concentration gradients within the cell as shown in Fig. 3. Systems displaying convective flow are referred to by Schachman (17). The convection observed in this study was tentatively attributed to an aggregation phenomenon inherent in the mucoprotein preparation and not to mechanical disturbances. This phenomenon has not been observed with other systems analyzed with the same instruments. Analyzing the mucoprotein over a pH range of 2.0–8.6 with buffers ranging from 0.05 to 0.5 ionic strength and at different temperatures did not diminish the disturbance. However, if the mucoprotein was analyzed at lower centrifugal speeds (20,410–31,410 r.p.m.), the convective flow was minimized and the sedimentation of the mucoprotein progressed as shown in Fig. 4. Under the conditions of these experiments, the mucoprotein had a constant S^0_{20} of 4.8. The anomalous behavior of the mucoprotein in high gravitational fields must be a function of the structural character of this complex. Additional investigations will be necessary to explain this phenomenon.

The diffusion constant of the mucoprotein was determined by the technique of double diffusion of antigen and antibody in agar gel[4] as described by Allison and Humphrey (18). The D_{20} was calculated to be 3.8×10^{-7} sq. cm./sec.

Assuming a partial specific volume of 0.75 and using the physical constants, S^0_{20} and

[4] Immunochemical characterization of the mucoprotein is presented in the following paper.

D_{20}, described above, the molecular weight of the complex was calculated to be 123,000.

The chemical composition of the mucoprotein is presented in Table I. Quantitative paper-partition chromatography indicated that mannose and glucose were the only hexoses present in the carbohydrate moiety of this substance. The value obtained for total reducing sugars compares favorably with the total hexose and hexosamine. The hexosamine was not identified.

A nondialyzable fraction, which accounted for the major portion of the carbohydrate, was isolated by digesting the mucoprotein with papain and purifying the hydrolyzate over columns of Sephadex gels. The fraction contained 32.1% hexose, 22.4% hexosamine, and 13.6% sialic acid. These results indicate that the carbohydrate moiety of the mucoprotein is a relatively large polymer because units of mono- or oligosaccharides would have been split off with protein fragments during enzymic digestion and lost in the dialyzate.

The ash content of the mucoprotein was 6.12%, which is quite high for a protein preparation. A copper content of 135–145 p.p.m. was routinely found associated with the complex.

The 2.1% lipid residue must be tightly bound to withstand the solvent extraction during preparation of the mucoprotein.

Sialic acid was released from the mucoprotein by neuraminidase[5] (in phosphate buffer, pH 5.9 at 35°C.) and by mild acid hydrolysis (0.1 N HCl at 37°C.). The progress of both the enzymic and acid hydrolyses was followed by measuring the liberation of N-acetylneuraminic acid by the method of Warren (13). The results are shown in Fig. 5. One unit of enzyme per milligram of substrate released a maximum of 65% of the total sialic acid by the end of 6 hr. The rapid release of this quantity of sialic acid indicates that it is present in the mucoprotein as terminal residues that are readily available for enzymic hydrolysis. After removing these residues with neuraminidase, the free-boundary electrophoretic mobility of the mucoprotein at pH 8.2 was decreased from −4.6 to −3.4 sq. cm./v./sec., indicating that the sialic acid contributed significantly to the negative surface charge at this pH. The remaining sialic acid was probably inaccessible to the enzyme due to structural or surface hindrances. The hydrolysis of sialic acid from the mucoprotein with dilute acid proceeded more slowly, but its release was essentially complete (97%) at the end of 72 hr.

[5] Isolated from *Vibrio cholerae*. Purchased from Behringwerke Akliengesellschaft, Marburg-Lahn, Germany.

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